

USPTO Serial No.: 10/549,518
Amendment Dated May 22, 2006
Reply to Notification to Comply dated April 27, 2006

Amendments to the Specification:

Please enter the enclosed paper copy of the sequence listing into the application.

Please replace paragraph [0003] bridging pages 2 and 3 of the application with the following amended paragraph:

[0003] To discover the molecular basis of cell adhesion and related cellular activities, several laboratories performed limited proteolysis to isolate cell-adhesive domains in fibronectin. Assignment of cell-binding activity has been based on assays measuring fibroblast attachment or spreading on fragment-coated substrates. Initially peptic cleavage of a 120 kD fibronectin cell-binding fragment and evaluation of its subfragments revealed a 108 amino acid 11.5 kD fragment that supported cell adhesion. From analyses of four synthetic peptides that together spanned the entire 11.5 kD fragment, one active site was localized to a 3.4 kD polypeptide at the C-terminus of the 11.5 kD fragment. Systematic testing of progressively smaller synthetic peptides, based on the 3.4 kD polypeptide sequence, subsequently identified a shorter peptide that now appears to represent the minimal active sequence. The active site contains the tetrapeptide Arg-Gly-Asp-Ser (RGDS) (SEQ ID NO:1). The RGDS (SEQ ID NO:1) sequence interacts with cell-surface fibronectin receptors, as demonstrated by RGDS (SEQ ID NO:1) competitive inhibition of fibroblast cell spreading on fibronectin-coated substrates. Soluble RGDS (SEQ ID NO:1) also inhibited the direct binding of radiolabeled fibronectin to fibroblastic cells in suspension. These competition studies indicated that the RGD sequence is critical for the cell adhesive function of the parent molecule.

Please replace paragraph [0064] on page 13 of the application with the following amended paragraph:

[0064] *In vitro* studies were undertaken to assess both the cell adhesive properties of surface immobilized 2-amino-6-[(2-amino-5{guanidino}pentanoyl) amino] hexanoic acid (AAGPAHA) peptidomimetic and the counteradhesive integrin antagonist activity of soluble AAGPAHA. AAGPAHA was covalently immobilized on dextran-coated tissue culture plastic utilizing methods earlier developed for cell adhesion peptides. Cell adhesion was on surface-immobilized AAGPAHA and compared to surface-immobilized peptide GRGDSP (SEQ ID NO:2). Other experiments were designed to assess the effect of soluble AAGPAHA on cell adhesion to GRGDSP (SEQ ID NO:2) to determine whether soluble AAGPAHA competitively inhibited integrin-RGD mediated cell adhesion. A similar experimental series was conducted to determine

whether soluble, integrin-binding GRGDSP (SEQ ID NO:2) competitively inhibited cell adhesion to surface immobilized AAGPAHA. Experimental methods and results are discussed below.

Please replace paragraph [0067] on page 14 of the application with the following amended paragraph:

[0067] For this study, we immobilized AAGPAHA and the peptide GRGDSP (SEQ ID NO:2) on dextran-coated tissue culture plastic dishes in a series of steps shown in FIGs. 2-4. Multi-well cell culture dishes were first surface-aminated by adsorbing poly-lysine (FIG. 2). Dextran was then immobilized on these surfaces (FIG. 3) using reported methods (Massia, Biomaterials 21:2753-61, 2000). AAGPAHA and GRGDSP (SEQ ID NO:2) were then surface-immobilized on dextran-coated tissue culture plastic (FIG. 4) using reported methods (Massia, J Biomed Mater Res 56:390-99, 2001). Dextran substrates were activated by oxidation of the glucose subunits (Glc) with sodium metaperiodate to convert Glc subunits to cyclic hemiacetal structures. Hemiacetal-containing subunits were then reacted with N-terminal amines of peptides forming an amine linkage between peptides and surface-immobilized dextran.

Please replace paragraph [0070] and the subtitle immediately before paragraph [0070] on page 15 of the application with the following amended subtitle and paragraph:

Covalent Coupling of GRGDSP (SEQ ID NO:2) and AAGPAHA.

[0070] Surface grafting of peptide GRGDSP (SEQ ID NO:2) and peptidomimetic AAGPAHA to dextran-coated substrates was achieved via previously reported methods (Massia, 2001). Dextran-coated substrates committed for peptide or peptidomimetic grafting were oxidized with 0.1 M sodium periodate, for 1 hr at room temperature to activate substrate surfaces for covalent immobilization of peptides (FIG. 4). Following surface oxidation, samples were rinsed with deionized water and then peptide or peptidomimetic stock solutions (0.1 mg/mL in 0.2 M dibasic sodium phosphate, pH 9.0) were added to each sample well. For 24 hrs a rocker table agitated the plates that were protected from light with a tin foil covering. Culture well substrates were decanted and rinsed with deionized water at the end of the 24 hr duration. Following peptide/peptidomimetic coupling, the substrates were incubated in dibasic sodium phosphate (0.2 M, pH 9.0) containing 0.1 M sodium borohydride, NaBH₄, to reduce Schiff bases formed and to quench any free unreacted aldehyde groups present (FIG. 4). The substrates were allowed to incubate for 2-3 hrs on the rocker platform. The substrates were rinsed with PBS and immediately were employed for *in vitro* experiments.

Please replace paragraph [0074] on page 16 of the application with the following amended paragraph:

[0074] Surface immobilization of dextran on tissue culture wells significantly reduced adhesion and spreading of all cell types (FIG. 5; 0.017 ± 0.03 % control). Virtually no cells were observed (FIG. 6A). Surface immobilization of AAGPAHA (the RGD mimetic) promoted extensive cell adhesion and spreading (FIG. 5; 105.9 ± 12.8 % control), comparable to substrates containing surface-grafted GRGDSP peptide (SEQ ID NO:2) (FIG. 5; 124.3 ± 4.1 % control). FIGs. 6B and 6C show thorough cell coverage of both protein surfaces. These results show that cells adhere to AAGPAHA as effectively as to the RGD peptide.

Please replace paragraphs [0076]-[0078] on page 17 of the application with the following amended paragraphs:

[0076] Soluble AAGPAHA peptidomimetic (0.1 mg/mL) completely inhibited 3T3 cell adhesion and spreading on surface-immobilized GRGDSP (SEQ ID NO:2) (FIG. 7; $0.08\% \pm 0.6\%$ control). This result indicates that soluble AAGPAHA bound to cell integrins competitively to inhibit integrin-mediated adhesion and spreading, *cf.*, FIG. 8C without soluble AAGPAHA and FIG. 8D with soluble AAGPAHA.

[0077] Similarly, soluble GRGDSP peptide (SEQ ID NO:2) (1 mg/mL) completely inhibited cell adhesion and spreading on surface-immobilized AAGPAHA (two left bars of the FIG. 7 graph; $5.7\% \pm 6.9\%$ control), *cf.*, FIG. 8A without soluble RGD and FIG. 8B with soluble RGD. This result indicates that cell adhesion to surface-immobilized AAGPAHA peptidomimetic substrates is integrin-mediated and can be inhibited by soluble integrin-binding GRGDSP peptide (SEQ ID NO:2).

Enzymatic Stability of RGD Peptide and RGD Peptidomimetic in Solution.

[0078] GRGDSP (SEQ ID NO:2) and AAGPAHA were mixed with trypsin solution in HEPES buffer (10 mM, pH=8) to a final solution of 0.4 mM peptide and 0.05 mM (0.12%) trypsin and incubated at 37 °C for 1 hr. In a control experiment, both peptides were dissolved in the same buffer without trypsin at the same final concentrations and incubated at 37 °C for 1 hr. Similarly, in another set of experiments, the same samples were incubated at room temperature overnight. At the end of the incubation period, trypsin activity was quenched by adding inhibitor; and the samples were analyzed by MALDI-TOF mass spectrometry to assess the extent of degradation.

USPTO Serial No.: 10/549,518
Amendment Dated May 22, 2006
Reply to Notification to Comply dated April 27, 2006

Please replace paragraph [0080] bridging pages 17 and 18 of the application with the following amended paragraph:

[0080] The results obtained from enzymatic treatment at 16 hrs, room temperature for RGD and AAGPAHA are shown in FIGs. 11 and 12, respectively. As can be seen in FIG. 11A, the RGD peak had an intensity of 1.5×10^4 in comparison to 3.0×10^2 after trypsinization (FIG. 11B). This is a massive decrease in the 588 nm GRGDSP (SEQ ID NO:2) mass peak, about 98% degradation of peptide. The FIGs. 12A and 12B mass spectra show mass peak intensity for AAGPAHA of 1.2×10^4 , which was the same after 16 hrs, room temperature treatment with trypsin (FIG. 12B), indicating that AAGPAHA is highly resistant to trypsin-mediated degradation.